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Formation of Sperm Entry Holes in the Vitelline Membrane of *Hydroides hexagonus* (Annelida) and Evidence of their Lytic Origin* ‡

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PLATES 154 TO 157

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ABSTRACT

Electron micrographs of inseminated eggs of *Hydroides hexagonus* previously had shown that in the immediate vicinity of the penetrating spermatozoön a small portion of the vitelline membrane regularly was absent, and it had been suggested that this area was a hole made by lytic activity of the individual spermatozoön during the course of its passage through the membrane. This deduction would receive support if it could be established that a sperm entry hole *does* form in living material.

During the present study a hole repeatedly observed and photographed in the membrane of living eggs was found to arise as the spermatozoön penetrated the membrane. Gently compressed eggs formed exovates only through this hole. The holes, and exovates, were not found except at sperm entry sites. It was concluded that this hole is the counterpart of the area from which the membrane is absent in the electron micrographs cited above, and that the spermatozoön makes this hole.

In an electron micrograph two spermatozoa which had penetrated the membrane at separate but closely neighboring points now occupy a single hole. It is argued that if each spermatozoön had displaced the membrane mechanically to make its hole, then there should be two holes, with a partition of membrane between them, but if each had eroded the membrane by applying lysin, a single hole should have formed as the eroded areas expanded and finally merged into one. The latter view agrees with the facts of the electron micrograph. It is concluded that lysis is the most probable means by which the individual spermatozoön makes its hole.

INTRODUCTION

The occurrence of holes in the egg membrane of fertilized eggs has been reported from time to time. For example, Mèves (18) in 1915 showed that a hole remained in an egg membrane of the mollusc *Mytilus* after the spermatozoön had penetrated the egg. His drawings were based on fixed and sectioned material. In 1956 Wada, Collier, and Dan (23) reported that they had observed an identical opening in the vitelline

membrane of living eggs of *Mytilus*. Austin (3) in 1951 described an elliptical hole or slit which the spermatozoön almost certainly had made in the zona pellucida of rat eggs although actual penetration of the zona by the spermatozoön was never observed. In 1954 Colwin and Colwin (14) observed pits which formed in the egg membranes at sites where spermatozoa were seen to enter living eggs of the enteropneust *Saccoglossus*, and later (13) counterparts of these pits were demonstrated in electron micrographs of sectioned inseminated eggs of this species. At the same time (13) it was also shown in electron micrographs that a portion of the vitelline membrane was absent from the area immediately surrounding the penetrating spermatozoön of the annelid

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Hydroides and it was suggested that this area was not an artifact but a hole normally made by lytic activity of the individual spermatozoön. The present study was undertaken in an effort to determine whether or not such holes could be found in living eggs. The holes, which were found, are shown in the following photomicrographs of successive views of sperm entry sites during the course of sperm entry. Brief abstracts of the findings have appeared elsewhere (11, 15).

Egg membrane lysins derived from sperm have been reported for an amphibian (16, 24, 25) and for several invertebrates (7, 17, 19, 21 to 23) and in 1948 Tyler (20) inferred that in molluscs and amphibians the egg membrane lysin would help the individual spermatozoön to penetrate the membrane barriers to the egg. At nearly the same time other investigators were making somewhat similar observations concerning mammalian sperm and fertilization (see Austin and Bishop (4, 5) and Chang (9) for reviews). Austin (1, 2), for example, in 1948 inferred that an enzyme probably enabled the individual mammalian spermatozoön to penetrate the cumulus oöphorus, and later Austin (3) and Austin and Braden (6) surmised that passage through the zona pellucida might also be effected by an enzyme. So far, however, the postulated lysin has not been demonstrated (5, 8). The above mentioned electron and light microscope studies in *Hydroides* and *Saccoglossus* (13, 14) gave special attention to sperm entry sites in the egg membranes; the evidence, though indirect, strongly suggests that the individual spermatozoön makes its entry hole by applying its own portion of lysin to the egg membrane. The electron micrograph presented below, showing two spermatozoa at nearly the same entry site, gives new evidence in favor of this suggestion.

Materials and Methods

The sperm and eggs used for this study were those of the annelid *Hydroides hexagonus*. Living material was studied on slides with supported coverslips. Photomicrographs were made using high dry and, chiefly, oil immersion objectives of conventional light (non-phase) microscopes and a Leitz Micro-Ibso apparatus with a $\times \frac{1}{3}$ tube and $\times 10$ ocular, and then enlarged photographically to the desired final magnification. Material for thin sectioning was fixed in 2 per cent osmium tetroxide in sea water for approximately 45 minutes and then dehydrated in increasing concentrations of ethyl alcohol. The material was infiltrated in three changes of a mixture of 85 per cent *n*-butyl and 15 per cent methyl methacrylate monomer containing

2 per cent luperco as catalyst. Polymerization was carried out in an oven at 63°C. Thin sections were cut with a Porter-Blum microtome and examined with an RCA model EMU-3C electron microscope. The electron micrograph was made at an original magnification of $\times 5,600$ and enlarged photographically to $\times 22,000$.

OBSERVATIONS AND DISCUSSION

Light Microscopy. Living Eggs.—The egg is enclosed by a well defined translucent envelope or vitelline membrane about 3 to 4 μ in thickness. Following insemination the spermatozoa seem to hit the outer edge and pause, then the successful one sinks slowly into the egg leaving a hole in the envelope.

The appearance of such a hole in one specimen is shown in Figs. 1 to 3. This egg had formed a fertilization cone. The hole, which was first observed before the cone had entered the envelope, remained clearly evident when the cone retreated (Fig. 2). Similar holes in other specimens are shown in Figs. 4 and 5. Figs. 6 to 8 show a hole during an earlier stage, while the sperm head was still passing through the envelope and, later, while the flagellum was passing through. This wide hole was easy to distinguish from the narrow flagellum but some holes were much narrower than this one. Often the narrower holes were nearly overlooked because the outline of the hole could be confused with an underfocus outline of the ingoing flagellum, as shown in Fig. 11. In this particular specimen, however, there was no real question as to the presence of the hole for the hole had been seen easily during the preceding stages (Figs. 9 and 10), and later was seen again (Fig. 12). Narrow holes showed well when the outline of the hole clearly failed to coincide with that of the flagellum (Figs. 9, 10, 12). Sometimes a hole was revealed briefly at the moment when some bent or deformed portion of the flagellum happened to pass through the envelope.

Occasionally the outer orifice of the hole was seen. Its rim appears as a flattened ellipse in the egg shown in Fig. 14. In this same egg Figs. 13 and 15 show the flagellum lying, respectively, close to the left side of the orifice, and approximately in the middle. Fig. 16 shows a specimen in which the orifice was viewed more nearly from above.

In some instances a hole was not seen in the envelope as the spermatozoön passed through. However, if the eggs were then gently compressed beneath the coverslip an exovate would form, and

it formed invariably at the site of sperm passage (Figs. 17 and 18). Fig. 19 shows a compressed polyspermic egg which had three exovates at three separate sites of sperm entry. Exovates of this kind were not formed when uninseminated eggs were compressed in this way. Clearly these exovates indicate sperm entry sites. But the specimen shown in Figs. 20 to 23 demonstrates more than this. In this specimen a hole was seen in the envelope as the sperm head passed through (Fig. 21). When, later, the egg was compressed an exovate was sent out through the same hole (Figs. 22 and 23). The presence of such an exovate at an entry site, then, can be taken to indicate the existence of an entry *hole* even when the hole itself fails to be seen. Similarly, it now seems likely that a fertilization cone of the kind that protrudes into the envelope (Figs. 24 to 26) also can be taken to indicate the presence of a hole in the envelope.

Twenty-eight living eggs of *Hydroides* were examined expressly with regard to the presence of a hole in the egg membrane at the site of sperm entry. The hole was seen in 19 of these and in 6 others its presence was indicated by the occurrence of a protruding cone or exovate. In two specimens the hole was listed as "probably present." One egg floated and could not be assessed as to the presence of a hole. Many uninseminated living eggs were also examined. In these, holes of the kind described above were never found. In no case was a specific hole kept in view up to the period of cleavage. However, from general inspection and from compression tests of cleaving eggs it appeared that the hole persisted at least throughout cleavage.

The findings concerning exovates through the egg membrane show some parallel to Austin's (3) observations of the exovates through the zona of the egg of the rat. While Austin obtained exovates at known sites of holes which were almost certainly *sperm entry* holes, some of the exovates described in the present report were at known sperm entry sites which were almost certainly *holes*. The appearance of the hole in the egg membrane as shown here is somewhat the same as that of the hole in the zona as described by Austin, though Austin has not dealt in any detail with the passage of the sperm head across the zona. No attempt will be made here to homologize these phenomena in the two species.

The present observations demonstrate that a well defined hole does indeed remain in the egg membrane of *Hydroides* after penetration by the

spermatozoön. *It is concluded that the hole is formed at the time of sperm entry.* As reported previously (13) electron micrographs show that in the vicinity of the penetrating spermatozoön there is an area from which part of the egg membrane is absent. *It is concluded that this area seen in electron micrographs is a counterpart of the hole seen in the membrane of the living egg.* Clearly the area is not an artifact but a consequence of sperm penetration.

Electron Microscopy.—The observations reported above do not in themselves reveal the way in which the entry hole is formed. On the basis of previous considerations (13) one would expect that the individual spermatozoön would apply lysin to the egg membrane rather than displace the membrane mechanically. Fig. 27 shows a section through two supernumerary spermatozoa which had penetrated the egg membrane at two different but not widely separated points. Typically (13) the principal material but not the outer border layer of the egg membrane is absent from the area nearest the invading spermatozoa. As happens frequently, microvilli project from the egg proper into this area (it is noteworthy that one of these appears to be in contact with the acrosomal region of one of the spermatozoa). It is to be noted especially, however, that the two spermatozoa which clearly had penetrated the membrane at separate sites *now occupy a single entry hole.*

If each spermatozoön had displaced the egg membrane mechanically then each should have formed a separate hole about itself; some portion of the intervening membrane should have remained present between the two spermatozoa and might even have become compressed between them. In the section, then, this material should be visible as a wall between two holes. Since there is no trace of such a wall in the common entry hole it seems most unlikely that mechanical displacement by the spermatozoa could have formed this hole.

If on the other hand the two spermatozoa had applied lysin to the egg membrane near their two points of entry, again two separate holes initially should have formed. But since in this case the cause of the holes would have been erosion, the intervening portion of the membrane should have disappeared as the two growing areas of erosion finally merged. The appearance of the present specimen, with its two spermatozoa in a single

large hole, is completely in keeping with this alternative.

It is concluded that lytic action was the most probable method of formation of this hole and, moreover, that the two spermatozoa were the sources of the lysin. It appears also, as previously observed (13), that this lysin does not dissolve the outer border layer. The mere existence of specimens such as this one gives new support to the suggestion (13) that the individual spermatozoön forms the hole in the egg membrane by local application of lysin. In further support of this interpretation may be mentioned recent findings that extracts of sperm of this species will dissolve the principal material of the egg membrane (10, 11). A detailed account of the action of these extracts on the egg membrane is presented in the following paper (12).

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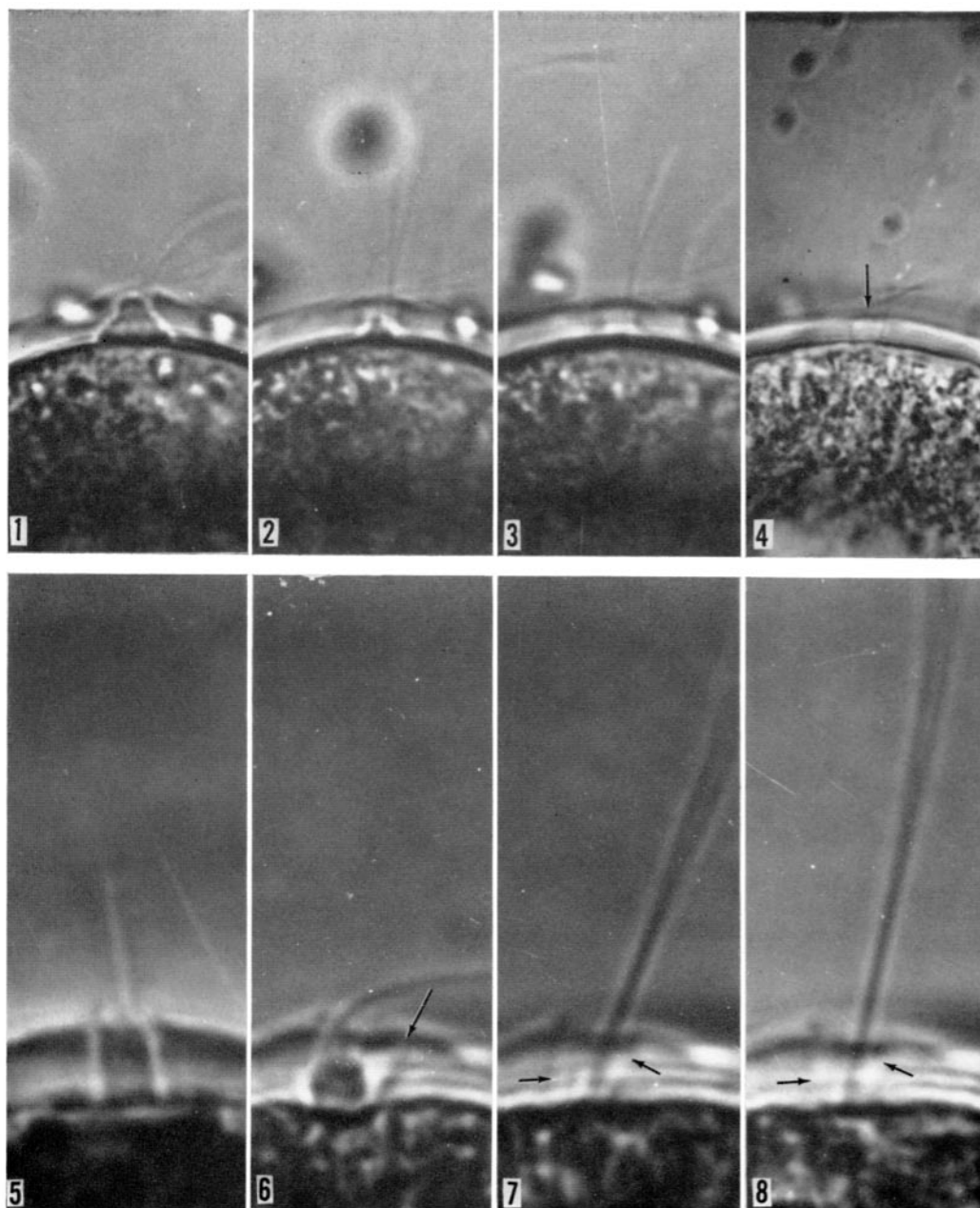
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EXPLANATION OF PLATES

All figures show sperm entry sites in the vitelline membranes of eggs of *Hydroides hexagonus*. All figures are photomicrographs of living eggs except Fig. 27, which is an electron micrograph.

PLATE 154

- FIGS. 1 to 3. Successive views of one egg. Flagellum of entering spermatozoön is visible. $\times 1600$.
- FIG. 1. Fertilization cone occupies much of hole left in vitelline membrane by entering sperm head.
- FIG. 2. Cone partly withdrawn. Outline of entry hole visible.
- FIG. 3. Cone withdrawn. Entry hole persists.
- FIG. 4. A slightly compressed egg. Arrow points to entry hole. Flagellum passing through right side of hole. $\times 1600$.
- FIG. 5. Entry hole in another egg; flagellum out of focus in area of hole. $\times 3700$.
- FIGS. 6 to 8. Successive views of sperm entry in one egg. $\times 3700$.
- FIG. 6. Part of sperm head still in vitelline membrane. Arrow points to right edge of entry hole.
- FIGS. 7 and 8. Flagellum passing through entry hole. Arrow: edge of entry hole.

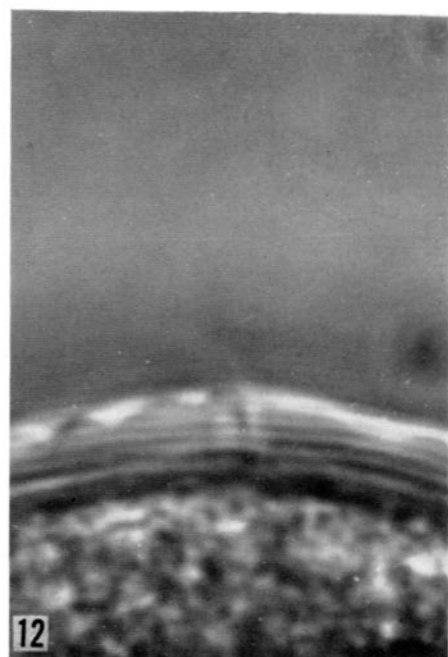
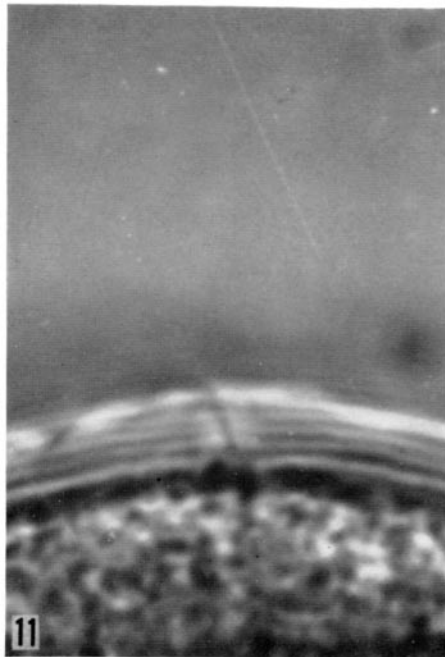
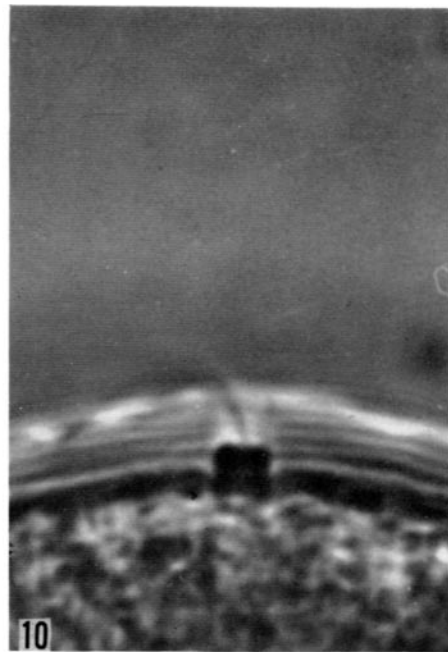
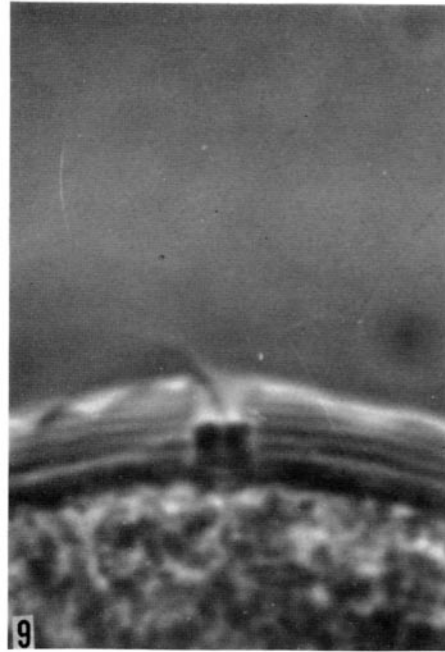


(Colwin and Colwin: Formation and lytic origin of sperm entry holes)

PLATE 155

FIGS. 9 to 12. Successive views of one egg. Entering spermatozoön leaves narrow hole in vitelline membrane. Flagellum crosses hole diagonally. $\times 3700$.

FIG. 11. Entry hole slightly out of focus and could be misinterpreted as underfocus outline of flagellum.



(Colwin and Colwin: Formation and lytic origin of sperm entry holes)

PLATE 156

FIGS. 13 to 15. Successive views of one egg. Positions of flagellum indicate orifice of sperm entry hole. Arrows point to flagellum. $\times 1600$.

FIG. 13. Point where flagellum crosses outer border of vitelline membrane indicates position of left side of orifice. Sperm head still visible in fertilization cone.

FIG. 14. Flagellum touches right side of orifice. Orifice appears as flattened ellipse at outer border of vitelline membrane.

FIG. 15. Flagellum crosses outer border of membrane near center of orifice.

FIG. 16. Another egg. Orifice is seen from nearly above. Arrow points to orifice. $\times 1600$.

FIGS. 17 and 18. Successive views of one egg. $\times 1600$.

FIG. 17. Entering flagellum indicates sperm entry site in vitelline membrane. Arrow points to outer edge of vitelline membrane.

FIG. 18. Under slight compression, exovate passes through membrane at sperm entry site. Arrow: portion of exovate protruding beyond membrane.

FIG. 19. Slightly compressed polyspermic egg with exovates marking 3 sperm entry sites. At right and center exovates, note flagella of entering spermatozoa. Arrow: outer edge of vitelline membrane. $\times 550$.

FIGS. 20 to 23. Successive views of one egg. $\times 1600$.

FIG. 20. Sperm head passing through vitelline membrane and starting to enter fertilization cone. Arrow points to tip of cone.

FIG. 21. Sperm head in cone. Hole remaining in membrane is partly obscured by tip of cone. Left side of hole lies directly below tip of arrow.

FIG. 22. Under compression, egg sends exovate into entry hole.

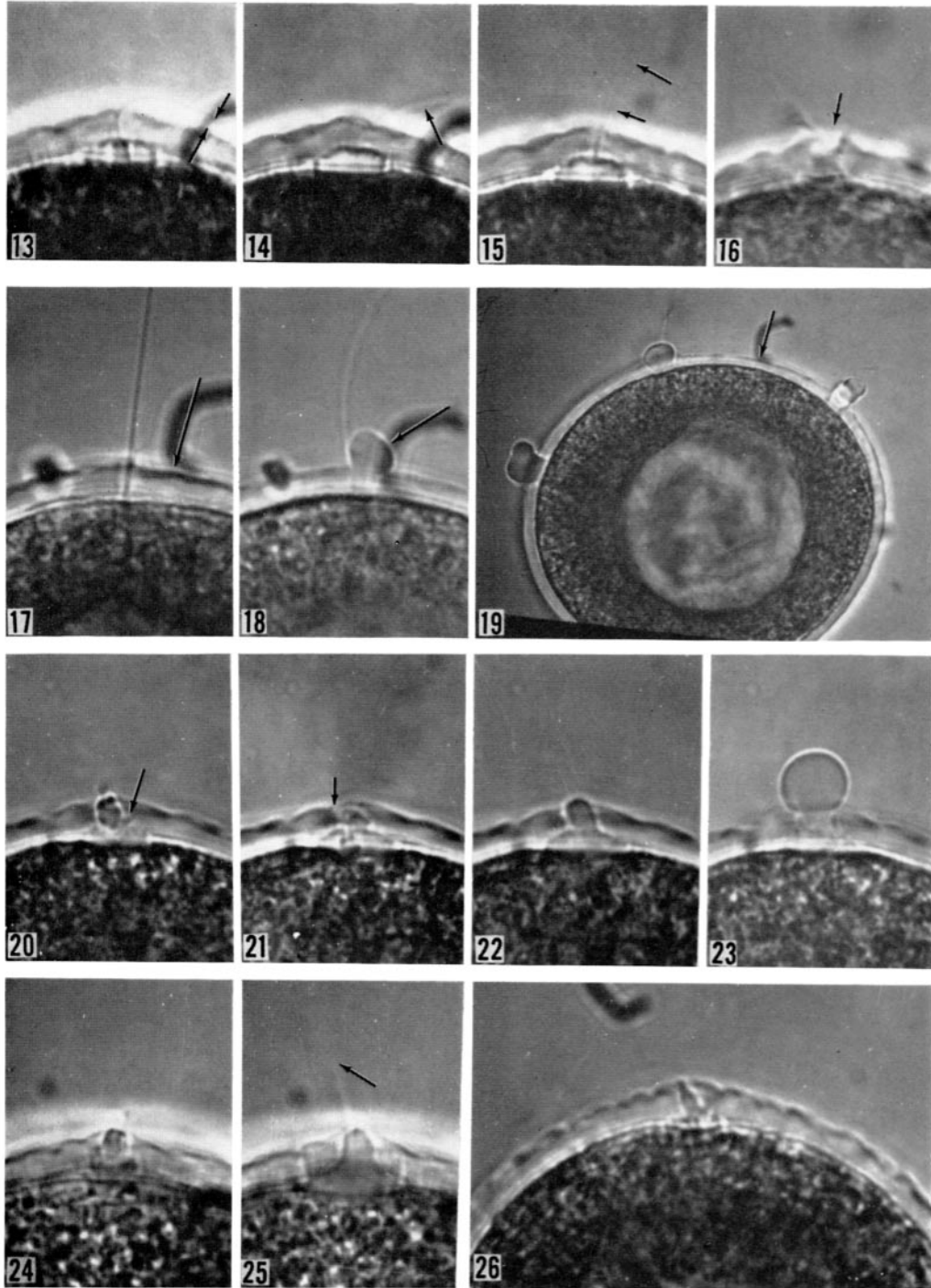
FIG. 23. Neck of exovate indicates extent of entry hole.

FIGS. 24 and 25. Successive views of one egg. $\times 1600$.

FIG. 24. Sperm head passing from envelope to cone.

FIG. 25. Projecting tip of cone indicates presence of hole in envelope. Arrow points to flagellum of still entering spermatozoon.

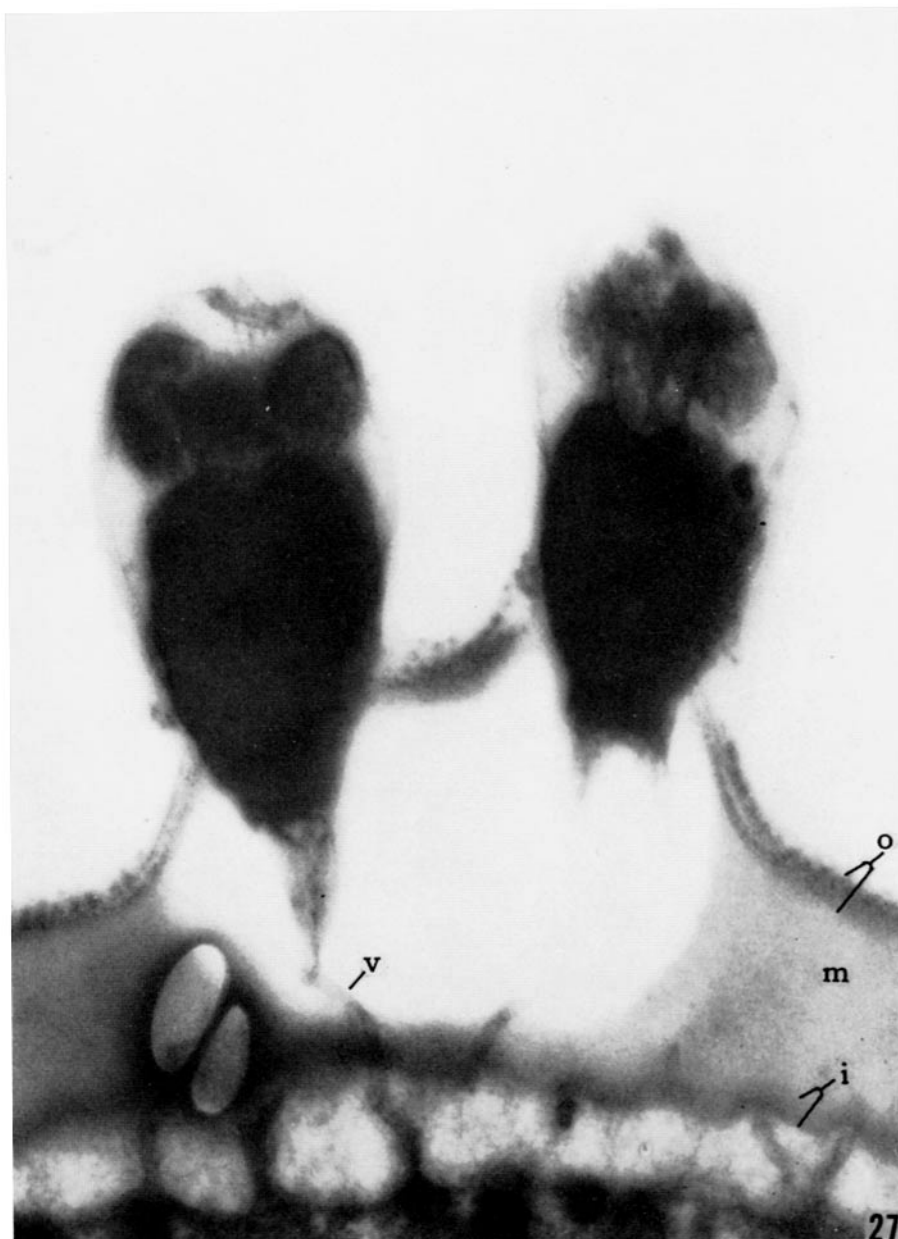
FIG. 26. Another egg with cone in envelope indicating presence of hole. $\times 1600$.



(Colwin and Colwin: Formation and lytic origin of sperm entry holes)

PLATE 157

FIG. 27. Section through two supernumerary spermatozoa which had penetrated at two separate points but now occupy a single hole in the vitelline membrane. (Note that the acrosome region of one spermatozoon appears to be in contact with a microvillus.) *i*: inner border layer; *m*: middle or principal layer of vitelline membrane; *o*: outer border layer; *v*: microvillus. Electron micrograph. $\times 22,000$.



(Colwin and Colwin: Formation and lytic origin of sperm entry holes)